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Molecular Genetic Analysis of Epstein-Barr Virus Cp Promoter Function

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The Cp promoter of Epstein-Barr virus (EBV) directs most transcription of the EBNA genes in lymphoblastoid cell lines. The functions of two control regions in the Cp promoter have been studied by construction of recombinant EBV strains containing specific mutations in these elements. Mutation of the RBP-Jk (CBF1) binding site reduced but did not completely abolish EBNA-2-dependent Cp activity in transient transfection assays. The same mutation in recombinant virus gave only a modest average reduction in Cp function, ranging from full activity to almost no activity in different isolates. Separate deletion of a 262-bp region containing glucocorticoid response elements had little effect in a transient assay but caused a fivefold increase in the steady-state level of Cp RNA in recombinant virus. The results indicate that other elements in addition to the intensively studied RBP-Jk site are important in determining Cp activity in the whole virus. Clonal EBV-infected cell lines expressed RNA from both the Cp and Wp promoters, but the level of Wp RNA did not simply compensate for changes in the level of Cp RNA. The levels of EBNA proteins varied much less than the levels of Cp and Wp RNA, suggesting other types of control in addition to initiation of transcription. A survey of RNAs derived from the internal repeat region of the virus indicated that gene expression from this region of EBV in lymphoblastoid cell lines is accounted for by the known transcripts.

The Cp promoter (BC-R2) of Epstein-Barr virus (EBV) is responsible for most EBNA mRNA expression in established lymphoblastoid cell lines (LCLs) and many group III Burkitt's lymphoma (BL) cell lines (4). Well-characterized LCLs that do not use Cp such as IB4 and X50-7 have been found to contain mutant viral genomes lacking Cp (19, 65). The regulation of the Cp promoter is important to the biology of EBV because it is the key control point distinguishing the immortalization (type III) latent pattern of gene expression, where Cp is active, from the latency type I gene expression seen in tumor cells (37) and circulating EBV-infected B lymphocytes in vivo (55), where Cp is inactive.

When a B lymphocyte is infected with EBV, another promoter, Wp, is responsible for the initial transcription of EBNA mRNAs, but after 3 to 6 days postinfection, a shift to use of Cp occurs (64). Since the activation of Cp does not occur when the EBV P3HR1 mutant strain infects B cells (62), EBNA-2 and EBNA-LP were presumed to trigger the activation of Cp, these genes being deleted or truncated in P3HR1. In transient transfection assays, EBNA-2 activates Cp transcription (21, 36, 52, 62), but EBNA-LP does not (36), so it was concluded that EBNA-2 activates Cp. Although there are examples of cell lines which exclusively use Cp rather than Wp (63), in most LCLs we have studied, Wp transcription declines rather than goes off completely when Cp is activated.

Deletion mapping of the Cp promoter region required for EBNA-2 transactivation in transient transfection assays showed that sequences between −433 and −245 relative to the start of transcription are important (52, 62). Detailed mutagenesis of the region from −400 to −330 identified sequences around −375 as essential for the EBNA-2 response (21). These experiments used an enhancer assay in which this region of Cp was tested for its ability to activate a minimal simian virus 40 promoter, the Cp elements being cloned far away from the simian virus 40 promoter. DNase I footprinting and gel retardation assays with extracts of infected cells revealed proteins (termed CBF1 and CBF2) that bind the regulatory region (21, 28, 62). The CBF1 complex contains EBNA-2, and a cell protein mediates the DNA binding of EBNA-2 in this complex (28). When the cell protein was cloned (28), it was found to be identical to the previously isolated RBP-Jk protein (29); it now appears that RBP-Jk is involved in all of the sites of EBNA-2 transactivation that have been studied so far (14, 27, 30, 60). RBP-Jk is considered to be a repressor of transcription on the basis of its ability to repress model promoter constructs (6, 18) and homology to the *Drosophila* repressor Su(H) (12). The powerful activation domain of EBNA-2 (5) is brought into the vicinity of the promoter through EBNA-2 binding to RBP-Jk, so both activation by EBNA-2 and loss of the repressive effect mediated through RBP-Jk may be important in the gene regulation (18). Although the functional properties of RBP-Jk and EBNA-2 have been studied in detail in other EBV promoters and in artificial promoter constructs (16, 22, 27, 28, 30, 60), the effect of specifically mutating the RBP-Jk binding site (CBF1 site) in the Cp promoter has not been reported. It was therefore of interest to test this by mutagenesis and to assess whether activation at Cp works via EBNA-2 transactivation or relief of the repressive function mediated through RBP-Jk.

There are clear examples in the analysis of cell gene expression in which promoters behave differently in transient transfection assays from the way they behave in their natural context in the genome. There are many possible reasons for this, including chromatin structure and long-range effects from dis-

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tant genetic elements omitted from the constructs used in transient transfection assays. It was notable in earlier studies of the Cp promoter that transfected Cp promoter constructs in EBV-positive cell lines sometimes displayed activity different from that of the endogenous viral Cp present in the cell line (59, 64). In addition to transient assays, we have therefore also tested the effect of mutating Cp promoter sequences in recombinant EBV strains in which precise replacement of nucleotides or deletion of sequences is possible in the context of the whole virus. This mutational analysis of Cp regulation with recombinant viruses became possible when it was found that the Cp promoter is nonessential for EBV immortalization (53), permitting isolation of Cp viral mutant strains.

In addition to the effects of EBNA-2 on Cp, regulation of Cp reporter constructs by glucocorticoids via glucocorticoid response elements (GREs) located about 900 bp upstream of the transcription start has been observed (26, 44, 45). We previously found that group I BL cell lines, in which Cp is inactive, have a much lower level of functional glucocorticoid receptor (GR) than group III BL cell lines or LCLs, in which Cp is active (48). In addition, the silent Cp in a group I BL cell line could (at low efficiency) be activated by overexpression of GR in the cells (48). However, an antagonist of glucocorticoid function, RU 486, had no effect on EBV immortalization of B lymphocytes, and the resulting cell lines used Cp normally (20), so the significance of the GREs during EBV immortalization is unclear. We have therefore tested the consequence of deleting the GRE region from the viral genome.

Cp can be deleted from the virus without loss of immortalizing capacity (53), and Wp may substitute for it in the resulting LCLs (52a). We have measured Wp transcription in the Cp mutant lines described here to determine whether Wp is also the functional EBNA promoter in these cells. The Cp promoter was originally identified as a result of isolation of cDNAs containing parts of the C1 exon (3). The Wp promoter was initially mapped by in vitro transcription assays of the major internal repeat IR-1 (57) and subsequently through cDNA analysis (40, 50). In neither the Cp mapping nor the Wp mapping was a systematic search made for other transcription starts for the EBNA mRNAs. There have been some hints that there might be other promoters; for example, Raji cells express the normal complement of EBNAs found in a group III BL cell line but have been reported to use neither Cp nor Wp (4, 63). The Fp and Qp promoters are located 125 kb upstream of EBNA-2 and EBNA-LP and are not thought to be able to express these proteins (39, 42, 43, 49). Also, the RNase protection or S1 nuclease analyses of Wp frequently show full-length protection of the probes in addition to the Wp initiation (47, 62), suggesting the presence of RNA initiated upstream of Wp. Furthermore, cDNA clones inconsistent with the known EBNA RNA structures were isolated by screening an expression library made with LCL RNA (58). We have therefore also surveyed the whole IR-1 region of wild-type (B95-8) EBV for novel RNA structures and compared the relative use of Cp and Wp and expression of the EBNA proteins in the viral recombinants.

MATERIALS AND METHODS

Cell lines and culture conditions. P3HR-1 clone 16 cells (obtained from George Miller, Yale University) contain a B-type EBV with a deletion of the region encoding EBNA-2 and part of the EBNA-LP genes, rendering it replication competent but transformation defective (35). B95-8 is a marmoset B-cell line immortalized with A-type EBV (32). DG75 is an EBV-negative BL cell line (2). Jijoye cells are derived from a BL (17) but display a group III phenotype. IB4 is an LCL derived from infection of umbilical cord blood B cells with B95-8 EBV (24). Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood purchased from North London Blood Transfusion Service. Blood was diluted two times in ice-cold phosphate-buffered saline (PBS) and passed through a Ficoll-Hypaque (Pharmacia) gradient. The mononuclear cells were depleted of T cells by 2-aminoethyl isothiouronium bromide-treated sheep erythrocytes. All cells were maintained in RPMI 1640 medium (Gibco) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated fetal calf serum.

Cosmid and plasmid constructs. The BZLF1 expression plasmid pSVNaeZ and the pDVcosA2 and SalA cosmids have been described previously (25, 54). The EBNA-2 expression construct, pSV-EBNA2 (8), an empty expression vector, pJ3Ω (34), the RBP-Jk expression plasmid (16), and the β-galactosidase expression plasmid pSV-β-Gal (Promega) have also been described.

The RBP-Jk site mutation was created as follows. A *Kpn*I-to-*Bam*HI fragment of B95-8 *Bam*HI C (10312 to 13215) was inserted into *Kpn*I-*Bgl*II-linearized pSP72 (Promega). A region including the RBP-Jk binding site (10946 to 11176) was amplified by PCR from an M13 clone template (362.BC, from the EBV sequencing program [1]) with a mismatched primer (G4089) to introduce GT GAATTC in place of GTGGGAAA. The PCR product was digested with *Dra*III and *Sac*I to produce a 130-bp fragment containing the mutated RBP-Jk binding site, and this was cloned into the *Dra*III-*Sac*I-digested pSP72 (10312 to 13215) plasmid. A *Kpn*I-*Bst*XI fragment (10312 to 12021) from this construct was then used to replace the corresponding *Kpn*I-*Bst*XI fragment in the SpH cosmid, which contains the *Spe*I-to-*Hin*dIII fragment of B95-8 EBV DNA (nucleotides 8668 to 48039). The SpH cosmid was constructed by digestion of the *Sal*I A BCRF1 cosmid (53) with *Hin*dIII, removal of EBV sequences 3′ to the *Hin*dIII site, ligation, insertion of an *Spe*I adapter at the 5′ *Sal*I site, and removal of EBV DNA from the converted *Sal*I site to the *Spe*I site at nucleotide 8668. The SpH cosmid contains an *Xba*I site inserted into the *Sal*I A BCRF1 cosmid at position 10023 (53). The *Spe*I-*Hin*dIII fragment (8668 to 48039, B95-8 coordinates) was then used to replace the corresponding fragment in the SalA cosmid (which had been modified so that the *Hin*dIII site at 48039 was unique) to produce SalA-E2RE-mut.

The GRE deletion was made in the SpH cosmid by *Kpn*I cleavage (at 10312), *Bal* 31 nuclease digestion, and religation of the digested ends. A suitable deletion was identified by screening DNA from bacterial colonies by PCR across the deleted region. Sequencing of the deletion breakpoint showed that nucleotides 10221 to 10482 had been deleted. The *Spe*I-to-*Hin*dIII insert of this cosmid was used to replace the *Spe*I-*Hin*dIII fragment of the SalA cosmid. Cosmid DNAs were packaged with Gigapack II packaging extracts (Stratagene) and used to infect the XL1-Blue strain of *Escherichia coli* (Stratagene).

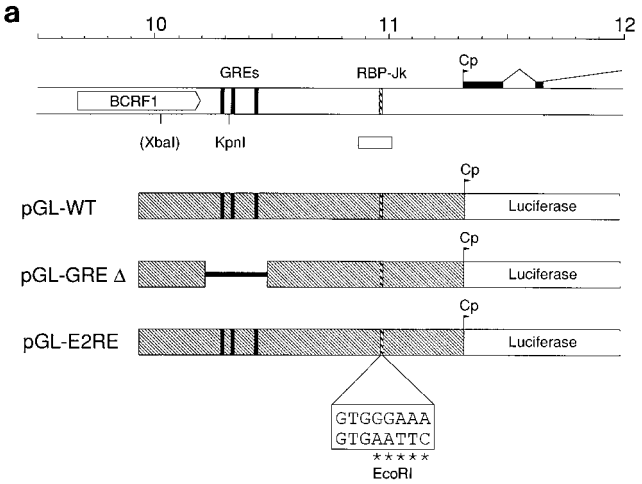
Luciferase constructs were generated by insertion of *Sau*3AI fragments (9911 to 11336) derived from wild-type, RBP-Jk binding mutant, and GRE-deleted mutant *Spe*I-*Hin*dIII cosmids into a *Bgl*II-digested pGL2 luciferase vector (Promega).

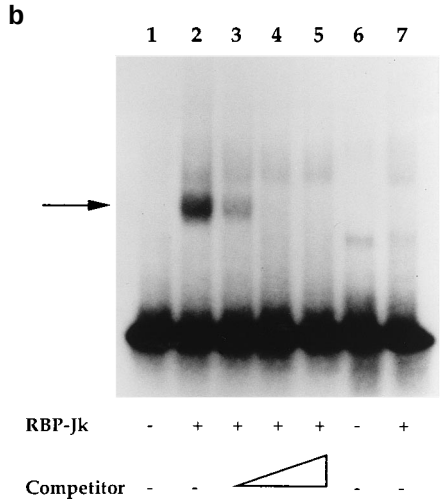
All plasmid and cosmid constructs were sequenced across the ligation sites and also at the point of mutation by the dideoxynucleotide chain termination method (41).

Transient transfections. All transfections were performed on at least three separate occasions with different preparations of DNA. For electroporation of DG75 cells, the culture was diluted two- to threefold with fresh medium 16 h prior to transfection. Approximately 10^7 cells were used per transfection in a total volume of 210 μl of ice-cold medium containing 2 μg of luciferase construct and 2 μg of pSVβ-Gal. The required amounts of pSV-EBNA2 and pJ3Ω were mixed, precipitated, and resuspended in water such that 5 μl was added to each cuvette. The cells were electroporated in a Bio-Rad Gene Pulser at 250 V (960 μF) and then incubated for 20 min at 37°C before transfer to 10 ml of conditioned growth medium. The cells were cultured for 24 h before assay. Cells were washed twice in ice-cold PBS, resuspended in 60 μl of reporter lysis buffer (Promega), and left at room temperature for 15 min. Cell debris was pelleted by centrifugation, and 20 μl of the supernatant was assayed for luciferase activity in an Autolumat (Berthold) with luciferase assay substrate (Promega). Twenty microliters of the cell extract was assayed for β-galactosidase activity (33).

Electrophoretic mobility shift assay. RBP-Jk protein was translated in vitro in the TNT rabbit reticulocyte lysate system (Promega) with T7 RNA polymerase. A reaction which incorporated [^{35}S]methionine into the product was performed in parallel to monitor efficient protein translation. Two microliters of nonradioactively labelled product was incubated with specific competitor oligonucleotides in BC100 buffer (10 mM Tris-Cl [pH 7.5], 50 mM KCl, 0.1 mM EDTA, 5% glycerol) and poly(dI-dC) (1 μg per reaction mixture) for 5 min at room temperature. A total of 50,000 cpm of ^{32}P-labelled probe was added, and the reaction mixture was incubated for a further 15 min at room temperature before fractionation on a 6% nondenaturing polyacrylamide gel. The probes were generated by PCR from wild-type and RBP-Jk site-mutated cosmids with the primers G5238 (GAGGCTAGTGTTTTAAAC, corresponding to B95-8 coordinates 10881 to 10898) and N5487 (TACTGTTCCAAAGATAGC, corresponding to B95-8 coordinates 11009 to 10991). The purified products were labelled with ^{32}P by the random priming method (10). The complementary oligonucleotides used for competition (N7544 [CACGCCGTGGGAAAAAAT] and N7545 [ATTTTT TCCCACGGCGTG]) spanned the RBP-Jk binding site in Cp corresponding to B95-8 coordinates 10953 to 10970 and were annealed by being heated to 95°C and being cooled slowly to room temperature.

Generation of recombinant virus. Fifteen micrograms of SalA cosmid DNA was cleaved with *Sal*I to release the EBV insert and coprecipitated with 40 μg of





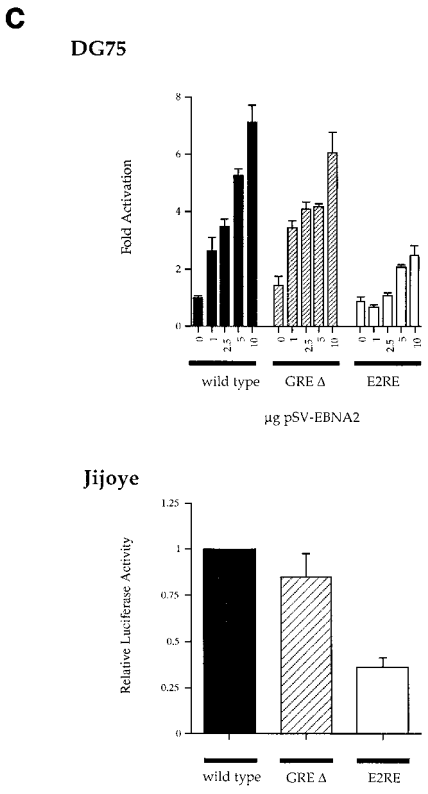


FIG. 1. (A) Luciferase constructs used in transient transfection assays are aligned to a map of the Cp region of B95-8 EBV (coordinates are in kilobases on the B95-8 map) (1). Each construct contains B95-8 sequences from nucleotides 9911 to 11336 cloned upstream of the luciferase open reading frame in pGL2 (Promega). pGL-GREΔ has a 262-bp deletion from 10221 to 10482 which removes the GREs and a *Kpn*I restriction site, and pGL-E2RE has a 5-bp mutation at the RBP-Jk binding site which introduces a novel *Eco*RI site. The position of the *Xba*I linker inserted in the BCRF1 open reading frame and the location of the probe used in the electrophoretic mobility shift assay (open box) are indicated. (b) Electrophoretic mobility shift assay of the wild-type Cp RBP-Jk DNA fragment (B95-8 coordinates 10881 to 11009 [lanes 1 to 5]) and the RBP-Jk site mutant (lanes 6 and 7). Double-stranded competitor oligonucleotide spanning the RBP-Jk binding site was added at concentrations of 6, 60, and 600 ng in lanes 3, 4, and 5, respectively. (c) (Top) Luciferase activities of the wild-type and GREΔ and E2RE luciferase constructs in DG75 cells with increasing amounts of pSV-EBNA2. Values were normalized for transfection efficiency and expressed as fold activation relative to that of the construct in the absence of EBNA-2. (Bottom) Luciferase activities of the wild-type and GREΔ and E2RE luciferase constructs in Jijoye cells. Values were normalized for transfection efficiency and expressed relative to that of the wild-type construct, which was assigned a value of 1.

pSVNaeZ plasmid. The DNA was resuspended in 50 μl of RPMI 1640 medium and used to transfect 8×10^6 P3HR-1c16 cells in a total volume of 350 μl. The cell-DNA mix was incubated on ice for 10 min prior to transfection in a Bio-Rad Gene Pulser at 220 V (960 μF). The cells were transferred to 10 ml of fresh growth medium and incubated for 3 to 4 days. The transfected cells were then pelleted, and intracellular virus was released by three cycles of freezing and thawing. The lysed cells were then combined with the growth medium and pelleted again. The supernatant was passed through a 0.4-μm-pore-size filter. A total of 2×10^7 to 5×10^7 PBMCs were infected by incubation in 2 ml of viral supernatant for 3 h with periodic shaking. Infected cells were plated at 5×10^4 cells per well in 96-well microwell plates in 150 μl of growth medium. One hundred microliters of fresh growth medium was added 7 days after plating, and then the wells were fed once per week.

PBMCs were infected with B95-8 virus in parallel with recombinant virus. Frozen 100× stocks of B95-8 virus (7) were used to infect 5×10^6 PBMCs and gave rise to numerous LCLs.

PCR screening of recombinant LCLs. LCL outgrowth was observed 3 to 5 weeks after plating and was characterized by macroscopic growth of cells in clumps and acidification of the culture wells. At this time, the cells were transferred to 24-well culture plates in a volume of 2 ml. Total cell DNA was prepared from 10^6 lymphocytes by lysis in 0.2× PBS, boiling, digestion with 1 mg of proteinase K per ml, and further boiling. DNA from 1.25×10^5 cells was used in a 30-cycle PCR mixture containing 200 μM deoxynucleoside triphosphates, 50 pmol of each primer, and 0.25 U of *Taq* DNA polymerase (Promega) in a 50-μl reaction mixture. The *Xba*I linker and the RBP-Jk site mutation were identified by cleavage of the PCR product by *Xba*I and *Eco*RI, respectively. The GRE deletion was recognized by a reduction in the size of the product from PCR amplification with primers spanning the deletion. The *Xba*I linker primers were 397L (TGATCCAATTCTACCTG) and 640M (TCAAATTCACTCATGGC), corresponding to B95-8 coordinates 9910 to 9927 and 10138 to 10122, respectively. The RBP-Jk site mutation primers were G5238 (GAGGCTAGTGTTT TAAAC) and M3803 (TCCTTGTCTCTATGCCAT), corresponding to B95-8 coordinates 10881 to 10898 and 11360 to 11343, respectively. The GRE deletion primers were G4830 (ATAATTGTTCGCAAGGCC [coordinates 10753 to 10736]) and 397L. The primer used to generate the RBP-Jk site mutation was G4089 (GTGTAAACACGCCGTGAATTCAAATTTATGGTTCAGTGCG), corresponding to B95-8 coordinates 10946 to 10985.

DNA isolation and Southern blotting. Genomic cell DNA was isolated from the nuclear fraction of cells that had been extracted twice with 150 mM NaCl–10 mM Tris-Cl (pH 7.5)–1 mM $MgCl_2$–0.1% Triton X-100 for cytoplasmic RNA preparation, as described previously (9). For Southern blotting, 10 μg of DNA was digested with *Bam*HI-*Kpn*I or *Bam*HI-*Eco*RI and then electrophoresed on a 1% agarose gel in Tris-borate-EDTA. The gel was denatured in 1.5 M NaCl–0.5 M NaOH for 20 min and neutralized in 1.5 M NaCl–1 M Tris-Cl (pH 8) for 40 min, and then the DNA was blotted to a nitrocellulose membrane. The filter was hybridized to appropriate ^{32}P-random-primed (10) DNA fragments generated by PCR. The *Bam*HI-*Kpn*I digests were hybridized to a mixture of two fragments which spanned B95-8 coordinates 9910 to 10138 and 11042 to 11497. The *Bam*HI-*Eco*RI digests were hybridized to the 11042 to 11497 probe only.

RNA extraction and RNase protection assays. Cytoplasmic RNA was prepared from the recombinant LCLs and from P3HR-1c16 and B95-8 cell lines as described previously (9). Riboprobes were generated by in vitro transcription of the

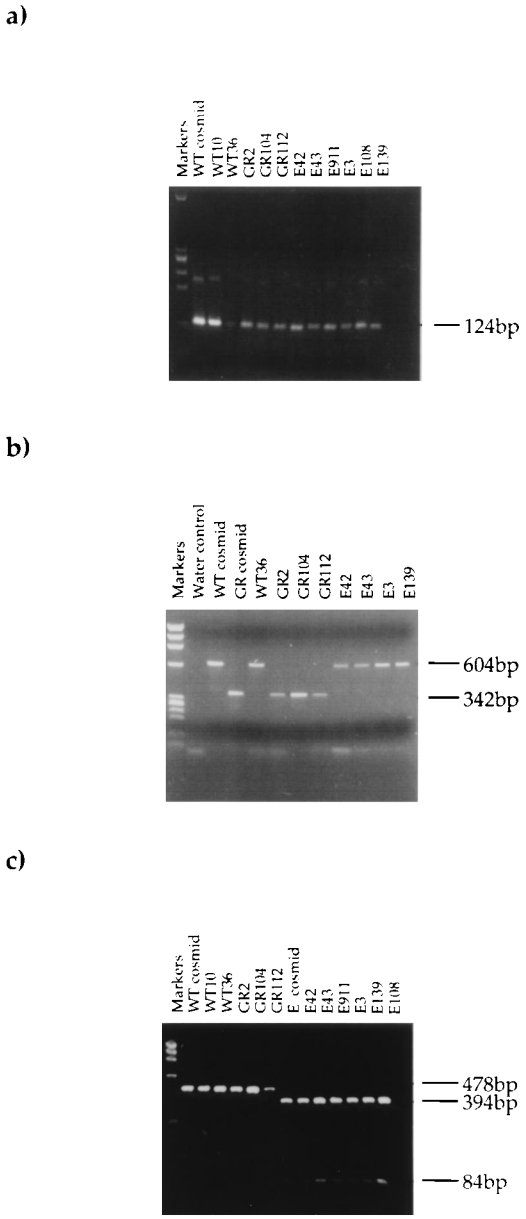


FIG. 2. Screening of recombinant cell lines by PCR. DNA from the cell lines indicated was amplified by PCR, and the products were analyzed by electrophoresis on 1.8% agarose gels stained with ethidium bromide. (a) Assay for the *Xba*I site from the B95-8 cosmid. (b) Assay for GRE deletion. (c) Assay for the *Eco*RI site in the mutated RBP-Jk site. Size markers were a *Hae*III digest of ΦX174 DNA. Sizes of products are indicated to the right.

vectors GEM3Z-406BC (45) (linearized with *Ava*II) for Cp usage and 5′ W0W1 (47) (linearized with *Sna*BI) for Wp usage with [α-^{32}P]rUTP (31). The γ-actin riboprobe was generated by cloning of the *Bam*HI-*Hin*dIII fragment of the pHF1 actin cDNA (15) into pSP64; the DNA was digested with *Mse*I prior to transcription of the riboprobe. The Cp and γ-actin probes were produced with SP6 RNA polymerase, and the Wp probe was produced with T7 RNA polymerase. RNase protection assays (Ambion) were performed with 50,000 cpm of riboprobe and 25 μg of cytoplasmic RNA as described previously (46). The other templates for the RNase protection probes were prepared by subcloning of the EBV content of M13 clones from the EBV sequencing project (1). The EBV sequence coordinates of the clones are as follows: clone 13, 13578 to 13933; clone 21, 14745 to 15083; clone 22, 12990 to 13219; clone 24, 12598 to 12944; clone 29, 14661 to 14880; clone 32, 15139 to 15345; clone 96, 13733 to 14074; clone 176, 14284 to 14651; clone 180, 13216 to 13529; clone 183, 12375 to 12563; clone 228, 14087 to 14482; and clone 229, 13350 to 13705. For each of these probes, the EBV insert was oriented such that for assaying rightward EBV transcripts, probes were made with T7 RNA polymerase on DNA linearized with *Eco*RI (except for clone 24, which required T3 RNA polymerase on DNA linearized with *Bam*HI). To assay leftward transcription, probes were made with T3 RNA polymerase on DNA linearized with *Bam*HI (except for clone 24, which required T7 RNA polymerase on DNA linearized with *Eco*RI).

Protein analysis. Cells were counted, washed once in PBS, and then solubilized in sodium dodecyl sulfate (SDS) sample buffer at a concentration of 10^6 cells per 50 μl. The samples were sonicated and heated at 95°C for 5 min prior to fractionation on a 10% acrylamide gel. The proteins were transferred to nitrocellulose filters and blocked in 5% dried skim milk in TBST (150 mM NaCl, 10 mM Tris-Cl [pH 7.5], 0.05% [vol/vol] Tween 20). The EBNA-2 monoclonal antibody PE-2 (Dako) was diluted 1:500 in TBST, and JF186 (11) and the RT human serum (from a healthy human donor, a kind gift of M. Allday) were diluted 1:10. A second layer of a 1:2,000 dilution of rabbit anti-mouse immunoglobulin (Z259 [Dako]) was used for the monoclonal antibodies, with a final layer of 1:10,000 horseradish perioxidase-conjugated goat anti-rabbit antibody (Dako). The filter treated with reticulocyte human serum was incubated with a 1:10,000 dilution of rabbit anti-human peroxidase-conjugated antibody (Dako). The antibody complexes were detected by enhanced chemiluminescence (Amersham). Comparable loading of each sample was confirmed by staining a duplicate gel with Coomassie blue.

RESULTS

Transient transfection assays of Cp promoter mutants. To determine the effects of mutation of the RBP-Jk and GREs in Cp, the plasmids depicted in Fig. 1a were constructed. The pGL-WT plasmid is a luciferase reporter equivalent to the previously studied −1.4CCAT plasmid (36). Two mutant plasmids were derived (Fig. 1a), one lacking the GREs because of a 262-bp deletion and the other containing precise replacement of four nucleotides in the RBP-Jk core binding site (GT GGGAA to GTGAATT). Extensive mutagenesis of the RBP-Jk site has been reported (27, 56) but this particular mutation has not been reported. DNA fragments containing the wild-type and the mutant RBP-Jk sites were therefore tested for their ability to bind in vitro-translated RBP-Jk in a gel retardation assay. The wild-type DNA was shifted by RBP-Jk, and this shift was specifically inhibited by a double-stranded oligonucleotide spanning the consensus site. The mutant DNA was not shifted at any concentration of RBP-Jk protein tested (Fig. 1b). The lack of binding of this mutant site has also been confirmed in a competition binding assay in which it was shown that the affinity of the mutated site for RBP-Jk was at least 100-fold lower than that of the wild-type site (26a).

The luciferase reporter constructs were electroporated into DG75 cells and the luciferase activity, corrected for transfection efficiency, was determined (Fig. 1c, upper panel). There was little difference between the basal activities of the promoters. When increasing amounts of an expression vector for EBNA-2 were cotransfected, the wild-type construct was activated up to sevenfold with 10 μg of pSV-EBNA2, consistent with earlier reports with similar constructs with another reporter system (36). Mutation of the RBP-Jk site reduced the EBNA-2 activation to about 2.4-fold, but the GREΔ construct still gave about a sixfold activation with EBNA-2. These results confirm that the RBP-Jk site is important for EBNA-2 transactivation of Cp in transient assays, although there was some residual activation in the RBP-Jk mutant, indicating that there may be further elements involved in the EBNA-2 response. The essentially unchanged basal activity of the RBP-Jk binding mutant indicates that the transcription repression function of RBP-Jk is relatively unimportant in this type of assay compared with the activation provided by the binding of EBNA-2. The GRE deletion seems to have little effect on the promoter in these transient assays in DG75 cells.

It is difficult to relate the amount of EBNA-2 that is expressed in a transient assay to that normally expressed in type III latent EBV infection, so the same constructs were also

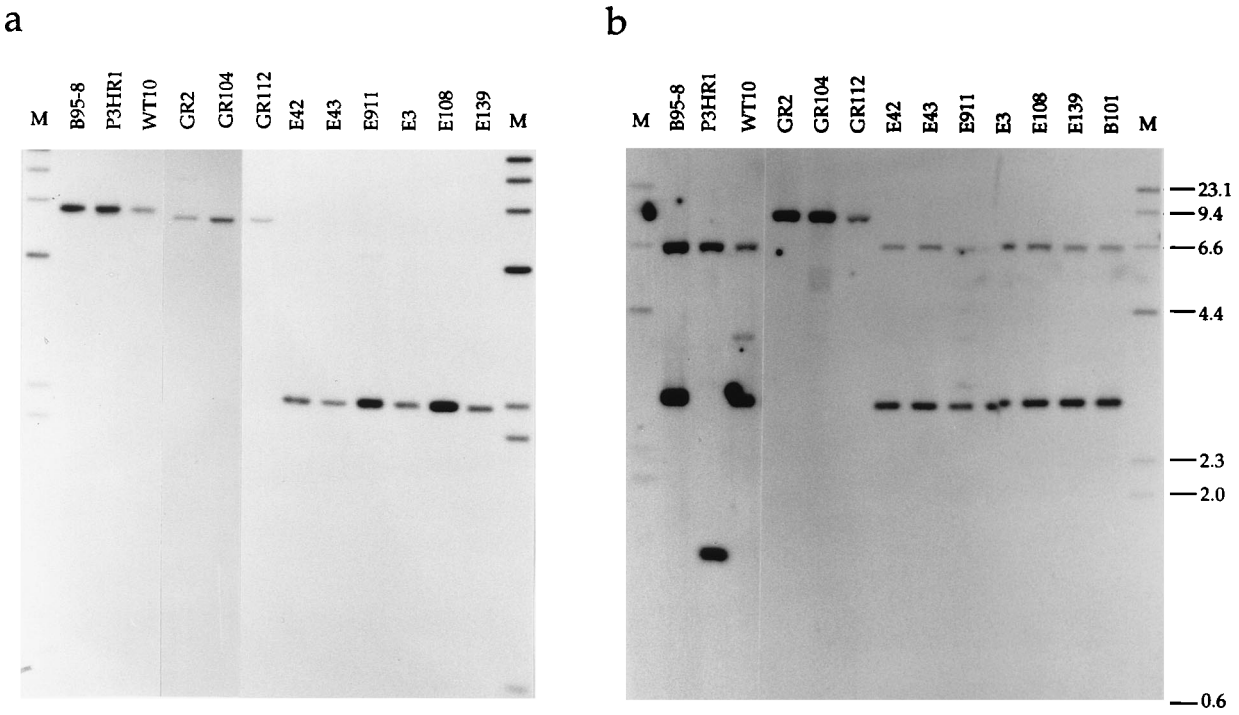


FIG. 3. Southern blot of DNA from LCLs containing recombinant EBV. (a) *Bam*HI-*Eco*RI digest probed with EBV sequences 11042 to 11497. (b) *Bam*HI-*Kpn*I digest probed with EBV sequences 11042 to 11497 and 9910 to 10138. Size markers were a *Hin*dIII digest of phage λ DNA and are indicated to the right in kilobases.

transfected into Jijoye cells, which express EBNA-2 (Fig. 1c, lower panel). Although the transfection efficiency of Jijoye cells was much lower than that of DG75, the relative pattern of activity of the plasmids was similar to that of the plasmids transfected into DG75 cells in the presence of pSV-EBNA2, supporting the conclusions derived from the DG75 transfections. The GR levels of DG75 have not been measured but would be expected to be intermediate, as seen in other EBV-negative cell lines (48). Jijoye cells express the high levels of GR characteristic of EBV-immortalized LCLs (48), so in this context, the GRE deletion seems to make little difference to the activity of the construct.

Isolation of Cp promoter mutants in recombinant EBV. The same GREΔ and RBP-Jk mutations were reconstructed in the *Sal*I A cosmid used previously for isolation of EBV strains mutated in the *Bam*HI C region (53, 54) by employing the strategy of linked transformation marker rescue from P3HR1 cells (54). All of these B95-8 cosmids were modified relative to the B95-8 EBV sequence by insertion of an *Xba*I linker (CTAGTCTAGACTAG) after position 10023 (within the BCRF1 gene [Fig. 1a]) (53); we have used this site to define the position of a crossover in the recombinant EBV strains isolated in this work. The wild-type cosmid and the two mutant cosmids were transfected separately into P3HR1 cells with an expression plasmid for the BZLF1 gene, which activates productive replication of the P3HR1 EBV. The EBV secreted from the cells, which contains a mixture of cosmid-P3HR1 recombinants and parental P3HR1 virus, was used to infect human B lymphocytes isolated from peripheral blood. P3HR1 is defective for immortalization (35), so only those cells which are infected with virus in which the EBNA-2 and EBNA-LP deletion in P3HR1 has been restored will result in outgrowth of LCLs. The lymphocytes were infected at a limiting dilution of virus and plated in wells so that only about 1/10 of the wells gave rise to outgrowth of an LCL.

DNA was prepared from the resulting LCLs and used to identify LCLs containing recombinant EBV with the desired mutations but free of contaminating parental P3HR1 EBV. The region containing the *Xba*I site in BCRF1 characteristic of the input cosmids was amplified by PCR and tested by restriction digestion for the presence of the *Xba*I site. LCLs which did not give a single PCR product that could be completely cut by *Xba*I were discarded (Fig. 2). The remaining lines should contain only recombinant EBV with a B95-8 Cp region which will be either wild type or mutated at the GRE or RBP-Jk sites. The presence of the GRE deletion was assayed by PCR amplification of the DNA spanning the sites (Fig. 2). The PCR fragment from the deletion-containing virus is shorter and lacks a *Kpn*I site located within the deleted region (Fig. 1a). The RBP-Jk site mutation (which introduces a novel *Eco*RI site [Fig. 1a]) was assayed by PCR amplification and restriction digestion of the products with *Eco*RI (Fig. 2).

The frequency of incorporation of the GRE and RBP-Jk mutations was estimated from the combined results of two recombination experiments in which a total of 1,152 wells were plated for each mutant type. For each mutation, about 7% of the wells gave rise to LCLs. For the RBP-Jk site mutation, 83 LCLs were obtained; 17 of them were singly infected with recombinant virus containing the mutation, 47 had only recombinant virus lacking the mutation, and a further 19 were coinfected with P3HR1. For the GRE mutation, 79 LCLs were obtained; 16 of them were singly infected with recombinant virus containing the mutation, 53 had only recombinant virus lacking the mutation, and a further 10 were coinfected with P3HR1. All of the singly infected mutant lines studied also had the *Xba*I marker (Fig. 1a) from the cosmid, indicating that the left-hand crossover point was to the left of this site; the lines subsequently analyzed for their Cp transcription properties were all from this group.

Representative lines from each group were then further

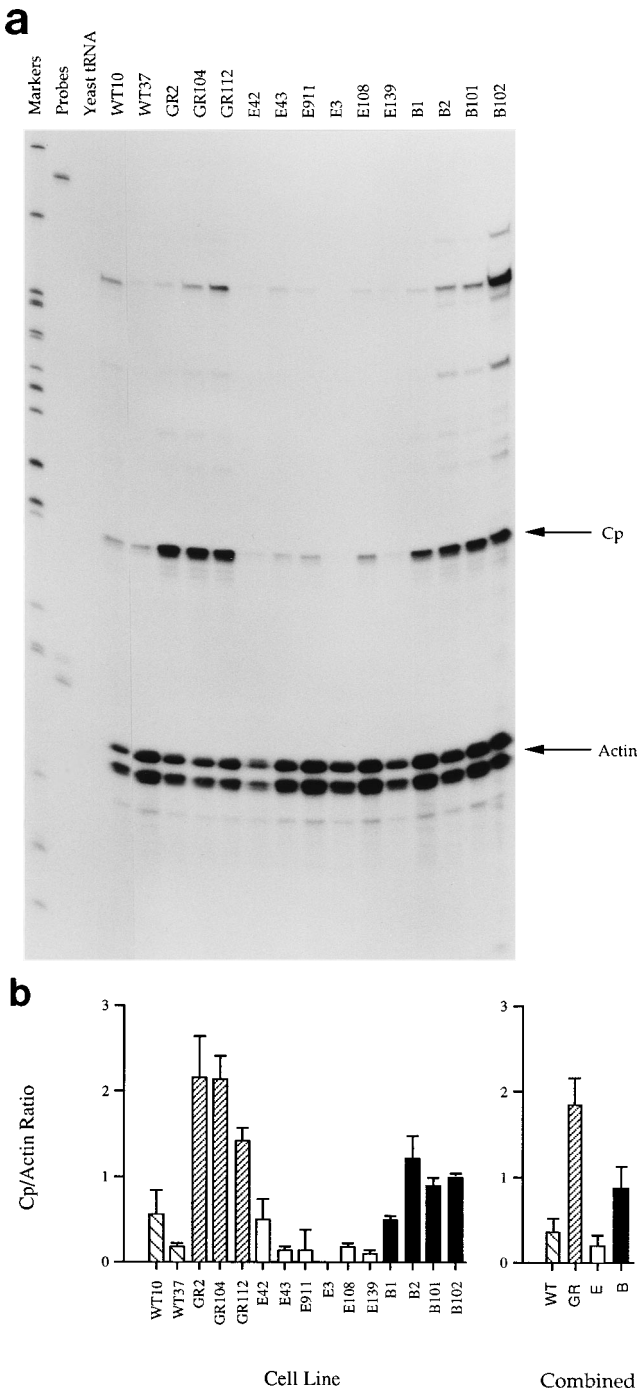


FIG. 4. (a) RNase protection analysis of Cp and γ-actin transcription in LCLs containing recombinant EBV. The specific protected fragments for Cp-initiated RNA (144 bp) and γ-actin RNA (110 bp) are indicated, and size markers were an *Msp*I digest of pBR322 DNA. (b) Quantitation of Cp mapping. The Cp and actin-protected fragments from Fig. 4a and other similar experiments were quantitated by densitometry of the autoradiograph and the results (left panel) are expressed as the ratio of Cp to actin RNA for the individual lines. Data for each mutant type were combined in the right-hand panel. WT, wild type.

analyzed by Southern blotting of the *Bam*HI C region to confirm the presence of the mutations and to ensure that they did not contain any substantial deletions or rearrangements. Examples of this are shown in Fig. 3. *Bam*HI-*Eco*RI digests confirmed the structures of the RBP-Jk site mutants, and *Kpn*I-*Bam*HI digests confirmed the structures of the GR mutants. An extra *Kpn*I site in *Bam*HI C of P3HR1 relative to B95-8 was identified at about 11600 (B95-8 genome coordinate), and this accounts for the shorter *Kpn*I-*Bam*HI fragment of approximately 1.3 kb in the P3HR1 track of Fig. 3b. This band is diagnostic of P3HR1, and its absence in the LCLs containing recombinant virus confirms the lack of coinfection with P3HR1 in these lines. These filters were subsequently probed with a series of EBV cosmids covering most of the remainder of the viral genome and with a probe for human actin (data not shown). The results showed no other changes detectable by this method in the EBV genome in the LCLs containing recombinant virus and a viral DNA copy number in all of the recombinant lines similar to that in the B95-8 LCL B101.

Transcription from Cp in LCLs containing recombinant EBV. Cytoplasmic RNA was prepared from the representative LCLs containing wild-type sequences, the GR deletion, and the RBP-Jk site mutation in which the recombinant viral genomes had been characterized. A series of LCLs made by B95-8 EBV infection of the lymphocytes used in each of the two recombination experiments quantitated above were also assayed for Cp transcripts (Fig. 4). RNase protection assays were used to measure levels of RNA initiated at Cp and a control cell RNA (γ-actin). Probes were devised which allowed simultaneous mapping of both RNAs in the same reaction mixture for accurate quantitation. The RNA correctly initiated at Cp gives a 144-nucleotide protected fragment from a 345-nucleotide probe (Fig. 4a), and the actin protection gives a 110-nucleotide band from a 117-nucleotide probe (Fig. 4a). The RNAse protection assays were performed between two and five times, and the results were quantitated by scanning of the autoradiographs and by PhosphorImager analysis. The amount of RNA correctly initiated at Cp was normalized relative to the actin signal, and the results of the multiple mapping experiments were combined to give the relative Cp RNA values and standard errors shown in Fig. 4b.

Compared with the wild-type recombinant EBVs, the GREΔ mutants gave about a fivefold higher level of Cp RNA. The RBP-Jk mutants gave a variable level of Cp RNA in different lines ranging from barely detectable to almost as much as those of the wild-type recombinant lines. The level of Cp RNA in the LCLs containing wild-type recombinant virus was about 2.5-fold lower than that in B95-8 LCLs; this might reflect long-range effects on Cp transcription in the recombinant viral genomes, which are chimeras of B95-8 (A-type) and P3HR1 (B-type) EBV or a different number of internal repeat units (IR-1) in the B95-8 virus from the recombinants. The most notable result of this mutagenesis analysis of Cp is that LCLs specifically lacking the RBP-Jk site nevertheless can show significant transcription from Cp, in some cases almost as much as that of the wild-type recombinant virus. In addition, deletion of a 262-bp region containing the GREs results in an increase in the steady-state level of Cp RNA, presumably as a consequence of increased Cp transcription.

EBNA protein expression in LCLs containing recombinant EBV. Since there were substantial variations in the level of Cp transcription in the various recombinant LCLs, the level of EBNA proteins in some of the lines was analyzed by Western blotting (immunoblotting) of cell extracts. The most extreme differences in Cp RNA levels were between the GREΔ and RBP-Jk mutant LCLs, so the analysis was restricted to these lines. The RBP-Jk site mutant lines E3 and E139, which have the lowest Cp RNA levels, also have the lowest EBNA-1 and EBNA-2 protein levels, but the difference in protein levels detected was much less than the difference in Cp RNA levels

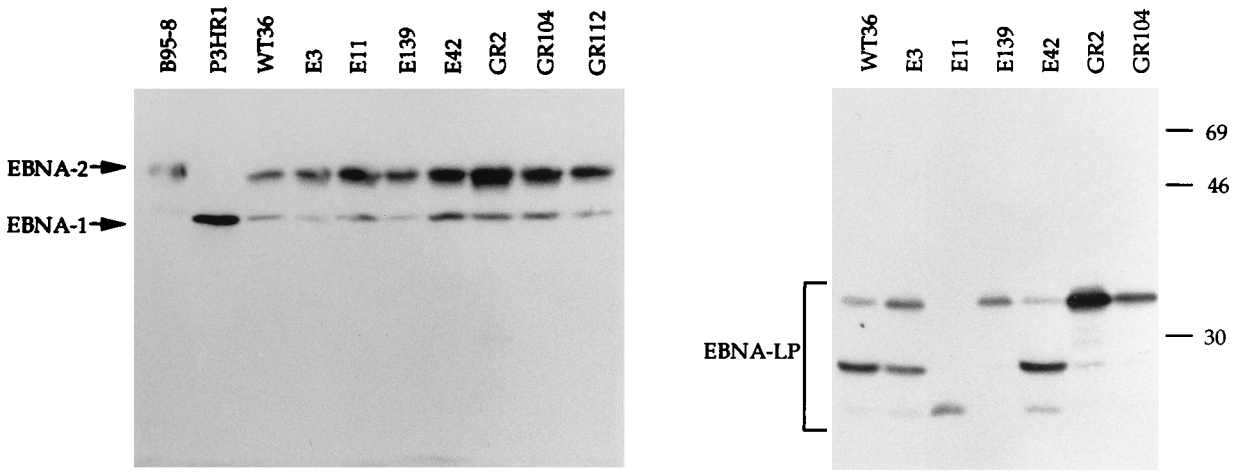


FIG. 5. Western blotting analysis of expression of EBNA-1 and EBNA-2 (left panel) and EBNA-LP (right panel) in LCLs containing recombinant EBV. Sizes of coelectrophoresed protein markers are indicated to the right in kilodaltons.

(Fig. 5). For example, GR2 has at least 50 times more Cp RNA than RBP-Jk site mutant E3, but there is only a modest difference in the protein level (estimated at about two- to threefold). A similar modest difference was seen in the levels of EBNA-LP (Fig. 5). This lack of direct quantitative correlation between the levels of EBNA proteins and Cp RNA suggests that either the steady-state EBNA protein levels in LCLs are determined more by protein stability than by mRNA level or that there are other initiation points for EBNA mRNAs in addition to Cp in these cells (for example, Wp). We have considered both the possibility of initiation at Wp and novel initiation sites for EBNA transcription in the internal repeat region.

Transcription initiation in the internal repeat region. The obvious expectation was that LCLs that used Cp at a very low level would use Wp to initiate their EBNA mRNA transcription, consistent with the previously reported mutually exclusive use of these promoters (63). Mapping transcription from the Wp promoter is complicated by the short length of the W0 exon (26 nucleotides) and alternative splicing at the W0W1 boundary. EBNA-LP is translated from RNA-spliced W0W1′ (50), and EBNA-2 is mostly translated from RNA-spliced W0W1 (61). Since the continued expression of EBNA-2 is essential for the growth of LCLs (23), we chose to measure the W0W1-spliced RNA. No clear inverse correlation was found between the level of Cp RNA and the level of W0W1-spliced Wp RNA (Fig. 6). For example, the RBP-Jk site mutant line E3 (which had almost no Cp RNA), had a slightly lower level of Wp-initiated RNA than GR2, which had one of the highest levels of Cp RNA. E139, which expressed modest levels of Cp RNA, had lower levels of RNA initiated at Wp than LCL E3 (Fig. 6). Since fewer than 1 in 10 wells gave rise to an LCL when initially isolated, it is highly likely that each LCL assayed in these experiments represents the clonal outgrowth of a single cell infected with EBV. The simultaneous transcription from Wp and Cp is therefore not likely to be due to a lack of clonality in the LCLs.

We also considered the possibility of initiation of EBNA transcription at other sites within the IR1 repeat region. We therefore tested for transcription initiation in the well-characterized B95-8 LCL, which uses Cp for its EBNA transcription (4). In addition, RNA from the IB4 cell line, which contains B95-8 EBV but uses Wp (40), was also assayed. A series of overlapping probes that covered the internal repeat region were developed (Fig. 7a). Single-stranded probes were made from each of these and used to test for both leftward and rightward transcription through this region. Mapping of rightward transcription (Fig. 7b) showed that all of the significant protected fragments corresponded to either full-length protection of the EBV content of the probes or were related to the known exons. For example, for probes 21 and 29, the major signal corresponds to the part of the probe covering exon W2 (marked a and b, respectively). In addition to the W2 signal with probe 29, there is a prominent band corresponding to the length expected for RNA running from the start of W2, through the splice donor at the 3′ end of W2, and through to the end of the probe (marked c). It therefore appears that a significant proportion of the RNA is not spliced at the 3′ end of W2. The band observed with probe 21 mapping at about 245 nucleotides would also correspond to this RNA. There are weaker protected fragments apparent in the RNase protection analysis which do not correspond to the known structures, but these appear to represent degradation of the full-length protected fragments through overdigestion.

The reciprocal set of probes made to test for leftward transcription showed little protection (Fig. 7c); the signals were much weaker than the specific protection signals seen in the rightward transcription mapping, and all of the leftward protected fragments corresponded to the full length of the EBV sequences present in the probes. At present, it is uncertain whether these signals indicate a very low level of specific leftward transcription or result from nonspecific transcription or residual viral DNA contaminating the RNA preparation, which might be detected with different levels of efficiency with the different probes.

DISCUSSION

Although there has been extensive analysis of the function of the RBP-Jk binding sites in model promoter constructs and at the LMP-1 promoter (14, 16, 22, 27, 28, 56, 60), the effect of specific mutation of the RBP-Jk site on transcription from the Cp promoter has not been studied directly. In transient transfection assays, we found that mutation of the RBP-Jk site greatly reduced but did not completely abolish EBNA-2 induction of Cp transcription (Fig. 1c). The similar results observed in Jijoye cells and DG75 cells transfected with EBNA-2 argue that the effects observed in DG75 reflect the activity of

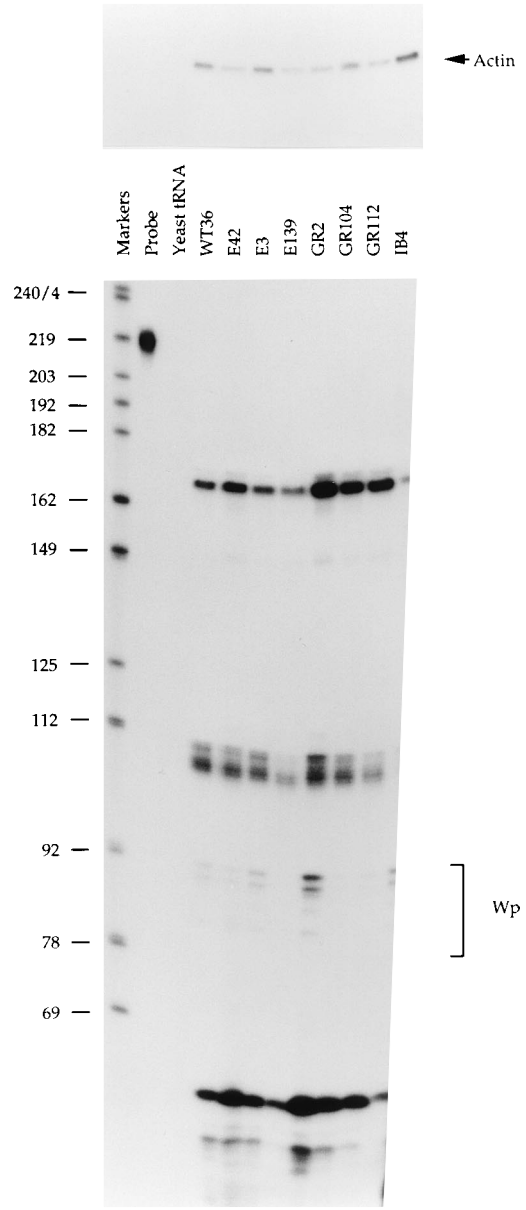


FIG. 6. RNase protection analysis of Wp transcription (bottom panel) and γ-actin (top panel) in LCLs containing recombinant EBV. The protected fragments corresponding to W0W1-spliced RNA initiated at Wp are indicated. Size markers were an *Msp*I digest of pBR322 DNA, and sizes are given to the left in nucleotides.

EBNA-2 in the normal physiological range of EBNA-2 expression. Since the mutation that was introduced into the RBP-Jk site reduced RBP-Jk binding by at least 100-fold, it is possible that part of the EBNA-2 activation of Cp might be through other elements in addition to the RBP-Jk site, as seen in the LMP1 promoter (22). It was also noteworthy that in the transient assays in DG75 cells shown in Fig. 1c, the basal levels of transcription in the absence of EBNA-2 were very similar in both the wild type and the RBP-Jk mutant. RBP-Jk has been shown to act as a transcriptional repressor (6, 18) and mutation of its binding site might therefore be expected to activate transcription. Since this was not the case, it seems that activation at Cp in this type of transient assay is mainly mediated by the binding of EBNA-2 (which carries its own activation domain [5]) rather than relief of a repressive function of RBP-Jk. There is clear evidence that the *Drosophila melanogaster* homolog of RBP-Jk, known as Su(H), acts as a repressor in vivo in the Notch signalling pathway (13), and it is possible that transient transfection assays are biased against displaying the effects of repressors, since a repressor might not be able to bind every reporter plasmid molecule in the time scale of the assay.

Since Cp has been shown to be nonessential for immortalization (53), it was possible to construct recombinant EBV strains carrying the same Cp mutations that had been tested in transient assays. In the whole virus, mutation of the RBP-Jk site resulted in only a small average reduction in the steady-state level of RNA initiated at Cp (Fig. 4b, right panel). The combined values, however, obscure a great variation in the level of Cp transcription in different LCLs containing the RBP-Jk mutant virus. In some lines, such as E3, Cp RNA was virtually undetectable, whereas LCL E42 had as much Cp RNA as the wild-type recombinant LCLs. It is likely that our experimental design, which requires the outgrowth of an LCL, imposes a considerable selection on recombinants to achieve EBNA expression. We cannot completely exclude the possibility that our RBP-Jk mutants that have high levels of Cp RNA might have second-site viral mutations which permit Cp transcription, but the similar yield of viral recombinants in all our experiments to those reported elsewhere (53, 54) argues against the need for selection of further mutations in the viral genome. It may be that in the context of the whole viral genome in an established LCL, other activating elements such as the EBNA-1-dependent enhancer in *ori*P (51) are able to activate Cp in addition to the likely role of EBNA-2.

It was notable that the level of Cp RNA in the LCLs containing wild-type recombinant EBV was about twofold lower than that in LCLs made with B95-8 EBV (Fig. 4B). Because of the incorporation of the *Xba*I marker site into the cosmids, we know that the recombinant viruses contain B95-8 EBV at least up to this point, which is 1.3 kb 5′ of the Cp transcription start. Downstream of Cp, the recombinants must be B95-8 at least through the EBNA-2 gene. The slightly reduced Cp activity in the chimeric viruses produced in the recombination (B95-8 is A type, P3HR1 is B type) might be due to long-range elements in the viral genome. Viral genome copy number did not seem to account for the difference (data not shown), but the copy number of the major internal repeat (IR-1) may differ between the recombinants and B95-8 EBV LCLs. Earlier work has suggested the presence of enhancer elements important for Cp activity in IR-1 (59). All of the cosmids used for the recombination experiments had the same number of IR-1 elements, so the wild-type recombinants provide a proper baseline with which to compare the activities of the RBP-Jk and GRE mutations.

The striking consequence of deleting the region containing the GREs was that Cp RNA was increased fivefold relative to that of the wild-type recombinant virus. This may indicate the presence of a novel negative element within the region deleted (10221 to 10482). Further, more-detailed mutagenesis will be required to determine whether it is the loss of the GREs or of another element within the deleted region that is responsible for this effect. Repression by GR through certain GREs has been observed (5, 38), but the GRE sequences in Cp correspond to those associated with activation, and we and others have argued elsewhere (26, 44, 45) that the GREs might be expected to have an activating effect on Cp. Computer searches of the sequence of the deleted region for the binding sites of known transcription factors did not reveal any other obvious

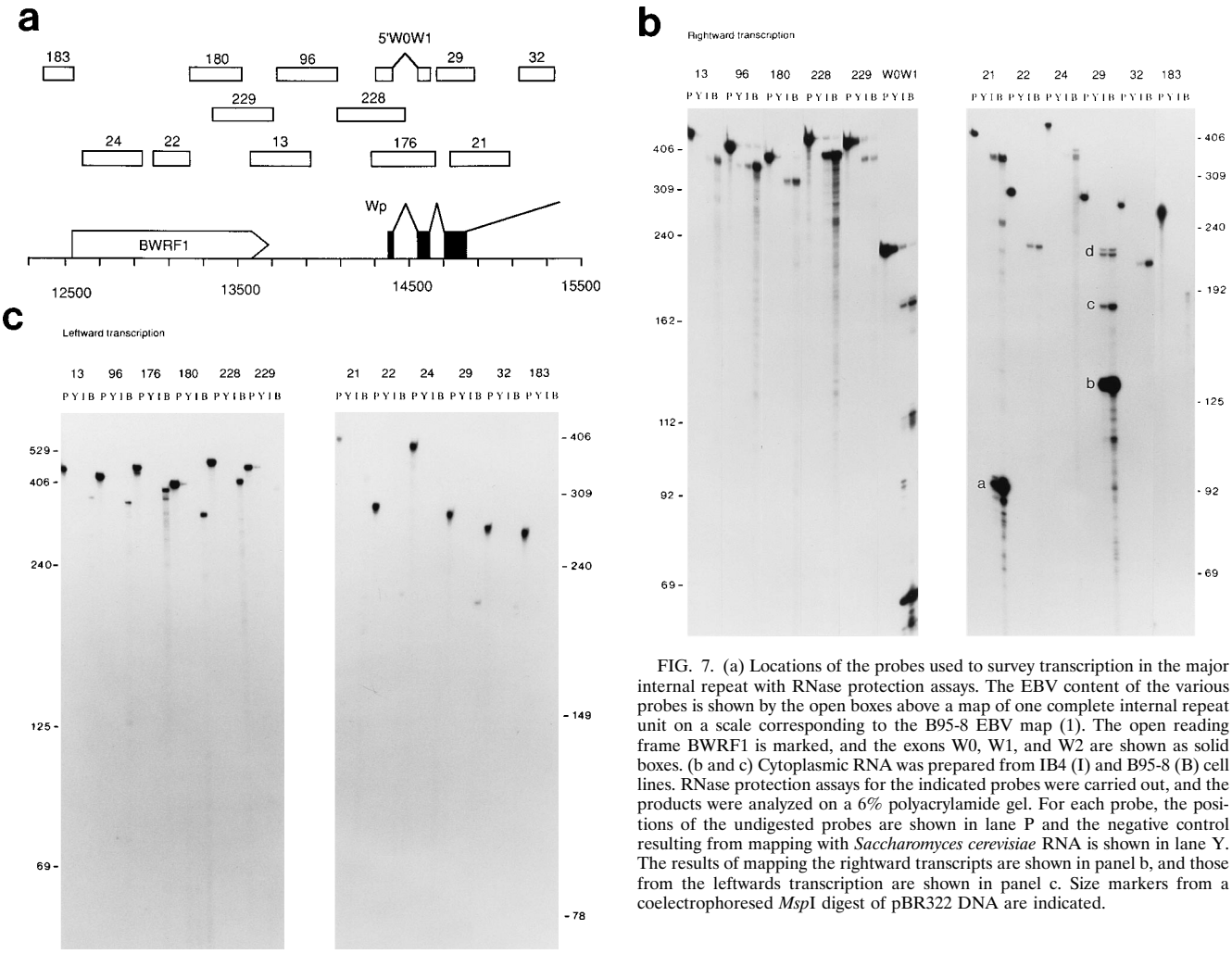


FIG. 7. (a) Locations of the probes used to survey transcription in the major internal repeat with RNase protection assays. The EBV content of the various probes is shown by the open boxes above a map of one complete internal repeat unit on a scale corresponding to the B95-8 EBV map (1). The open reading frame BWRF1 is marked, and the exons W0, W1, and W2 are shown as solid boxes. (b and c) Cytoplasmic RNA was prepared from IB4 (I) and B95-8 (B) cell lines. RNase protection assays for the indicated probes were carried out, and the products were analyzed on a 6% polyacrylamide gel. For each probe, the positions of the undigested probes are shown in lane P and the negative control resulting from mapping with *Saccharomyces cerevisiae* RNA is shown in lane Y. The results of mapping the rightward transcripts are shown in panel b, and those from the leftwards transcription are shown in panel c. Size markers from a coelectrophoresed *Msp*I digest of pBR322 DNA are indicated.

potential repressor binding sites, and there was no evidence for chance creation of a transcription activator site at the point of the deletion. It is possible that the activation is a consequence of *ori*P being closer to Cp in the GRE-deleted viruses; only 262 bp have been deleted, and *ori*P is over 2,000 bp away from Cp, but the possibility of increased *ori*P activation cannot be excluded. The constructs used in the transient assays contained sequences up to 1.4 kb upstream of Cp, but *ori*P was not present in those plasmids; the 262-bp GRE deletion had no significant effect in the transient assays in both DG75 and Jijoye cells.

Although there were large differences in the amounts of Cp RNA in the different lines, any differences in the amounts of the EBNA proteins were much smaller. For example, GR2 had at least 50 times more Cp RNA than RBP-Jk site mutant line E3, but there was only a two- to threefold difference in the protein level. We expected that EBNA transcripts from Wp would compensate for the variation in Cp RNA, since the use of these promoters has been reported to be mutually exclusive in LCLs (63), but in our hands, no simple relationship between the levels of Cp RNA, Wp RNA, and the EBNA proteins was found. The EBNA protein levels tended to be higher in the lines with more Cp or Wp RNA, but in LCLs, so long as there was some Cp or Wp RNA, the major determinant of EBNA protein levels was not at the level of transcription. The EBNAs are stable proteins, and this may be a more important determinant of the protein levels observed than the Cp and Wp RNA transcripts are. The Northern (RNA) blotting and cDNA sequencing data relating RNA transcribed from Cp and Wp to the EBNA mRNAs (3, 4, 40, 50) cannot exclude the possibility that much of the EBNA RNA might not be translated, so this remains another possible explanation of the discrepancy between RNA and protein levels.

The discrepancy between EBNA RNA and protein levels might also be explained if there were another promoter for the EBNA RNAs that has not yet been identified. We therefore searched the IR-1 repeat region for evidence of novel transcription initiation. We did not find any significant level of novel transcripts, in either the rightward or leftward direction. Our experiments did not reveal any novel specific transcripts that might correspond to the clones identified by Walls and Gannon (58) in an expression library from an LCL with sera from people infected with EBV. We conclude that the substantial full-length protection often seen with Wp mapping probes most likely represents transcription from the upstream Wp element running through to the next repeat with incomplete splicing, particularly at the 3′ end of the W2 exon as shown in Fig. 7b. Since we have only tested the RNA of established LCLs, our data do not exclude the possibility of another promoter being active very early during infection, when the full-length protection of Wp probes is particularly

strong (47), but there was no evidence in our experiments for novel EBNA transcripts in the B95-8 or IB4 LCLs.

This is the first report of the use of EBV recombinants to study an EBV promoter, and this method has revealed novel features of gene regulation in the virus. It will be desirable in the future to test other promoter mutants in the recombinant virus system, but the lengthy nature of the procedure used here would make it very time-consuming at present to analyze a large array of mutants. The system imposes a selection on the recombinants to permit cell growth and probably favors LCLs that produce wild-type levels of EBNA proteins. EBNA promoter switching from Wp towards Cp is the normal course of events in EBV infection (64), and we consider that by mutating the RBP-Jk site we have selected a variety of solutions by which the virus can achieve adequate EBNA expression. Some of these evidently involve Cp activity, and some do not. Nevertheless, the similar frequencies of outgrowth of LCLs transformed with the RBP-Jk mutant or wild-type recombinants make it unlikely that selection of a rare second-site mutation is involved, but rather there is sufficient redundancy of transcription factor elements in the viral genome to permit EBNA expression even without the normal RBP-Jk–EBNA-2 binding.

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